EXAMPLE 8

Ectopic Expression of Maize WUS to Induce Organogenesis

Using the genotype High type II as an example, embryos are isolated and cultured on 560P medium for 3-5 days. Twelve hours before bombardment these embryos are transferred to high osmotic 560Y medium. Expression cassettes containing the WUS cDNA are then co-introduced into the scutella of these embryos along with an expression cassette containing genes encoding selectable markers, such as Bar or Pat, or visual markers such as green fluorescent protein (GFP) or cyan fluorescent protein (CFP) using methods well described in the art for particle gun transformations. Twelve to 24 hours following bombardment, embryos are then transferred back to 560P culture medium and incubated in the dark at 26°C. After one week of culture these embryos are moved to 560R selection medium. Cultures are then transferred every two weeks until transformed colonies appear. It is expected that expression of WUS will stimulate adventive meristem (shoot) formation. This will be apparent when the cultures are compared to controls (transformed without the WUS cDNA or non-induced). Using either inducible expression cassettes, tissue specific promoters, or promoters of varying strengths it will be possible to control the levels of expression to maximize the formation of adventive meristems. Using either non-responsive genotypes or sub-optimal culture conditions with responsive genotypes, only the transformed cells expressing the WUS cDNA will form meristems and regenerate plants. For experiments in which WUS-induced shoot proliferation occurs via ectopic meristem formation, WUS can be used as a positive selective phenotype and no selection agent is required in the media. In this manner the WUS gene can be used as a positive selective marker (only the cells expressing the gene will form shoot meristems) and transformants can be recovered without a negative selective agent (i.e. bialaphos, basta, kanamycin, etc.).

Results

A. Ectopic Expression of maize WUS1 to Stimulate Organogenesis.

Using the maize genotype High type II, immature embryos were isolated, cultured and transformed as described generally in Example 7 substituting 0.6μm gold particles for tungsten and with the sample plate 7.5cm below the stooping screen. DNA was delivered using co-transformation, a method where introduced DNA's are normally integrated in a single locus. As a control, embryos were shot with a 1:1 mixture of plasmids, the first plasmid containing a ubiquitin promoter-driven green fluorescence protein (GFP) and a second plasmid containing a ubiquitin driven uidA gene (GUS). In the WUSCHEL treatment, the embryos were bombarded with a 1:1 mixture of plasmids containing the ubiquitin promoter driving expression of GFP (Ubi:GFP) and a plasmid containing the WUS1 DNA (SEQ ID NO: 32) driven by the maize In2 promoter (In2:WUS1). The In2 promoter is induced by auxin-like compounds and is weakly expressed on callus maintenance medium. Each treatment contained 175 embryos. Embryos were allowed to callus on 560P medium without selection. After approximately 3 weeks, GFP-positive tissue was visually selected under a fluorescent microscope and transferred to fresh medium. After 8 weeks colony numbers and size were recorded. In the control, 4 small events (<1 cm^2) were recovered along with 1 medium event (1-2 cm^2) for a total of 5 events. In the WUS1 treatment, the colonies were both more numerous and larger. In this treatment 6 small events (<1cm^2) were recovered along with 2 medium size events (1-2cm^2) and 3 large events (>2cm^2) for a total of 11 events. Plants were regenerated and some unusual phenotypes were observed during the plant regeneration process in the In2:WUS1 treatment, most notable were somatic embryos derived from root tips, a phenomenon observed with ectopic WUS expression in *Arabidopsis* (Zuo et al. (2002) Plant J. 30:349-359) One WUS1 event was observed with a highly unusual phenotype. In this event, ectopic earshoots and

leaf-like structures were formed in a radial pattern on the abaxile side of the vegetative leaves. This is a highly unusual placement for meristems in angiosperms but a similar phenotype has been observed in *Arabidopsis* plants over-expressing WUSCHEL (Lohmann et al. (2001) Cell 105:793-803). All other regenerated plants were normal, as expected, since the In2 promoter is not on in the absence of auxin-like chemicals.

B. Ectopic Expression of Maize WUS in an Inbred to Stimulate Organogenesis.

Immature embryos were transformed as described in Example 7 with media alterations done to adapt the procedure for use with inbred germplasm and visual selection. Using the maize inbred 581, a tissue culture recalcitrant line, immature embryos from greenhouse grown plants were isolated 12 days after pollination and cultured on 605J medium (a medium containing both full strength MS salts (macro and micronutient) and 0.6X N6 macronutrient salts plus additional B5 micronutrients, with a mixture of SH and Eriksson's vitamin, L-proline and casamino acids, silver nitrate, 0.3mg/l 2,4-D and 1.2mg/l Dicamba, 2% sucrose and 0.06% glucose, solidified with agar). The embryos were incubated in the dark at 28°C overnight. The embryos were then transferred to a high osmoticum medium similar to 605J with the addition of 15% sucrose prior to particle bombardment. Embryos were shot in a method similar to that in Example 7 substituting 0.6µm gold particles for tungsten. DNA was delivered using co-transformation, as noted above. As a control, embryos were shot with a 1:1 mixture of plasmid DNA's containing a Ubiquitin driven yellow fluorescence protein (YFP) and a plasmid containing a Ubiquitin driven uidA gene (GUS). In the WUSCHEL treatment the embryos were bombarded with a 1:1 mixture of plasmid DNA's containing the Ubiquitin promoter driving expression of YFP (Ubi:YFP) and a plasmid containing WUS2 (SEQ ID NO: 62) driven by the maize In2 promoter (In2:WUS2). Immediately following bombardment embryos

were transferred back to low osmoticum 605J medium. Each treatment contained 90 embryos. Embryos were observed 3 days after bombardment and differences were observed between the treatments.

In the control treatment, hundreds of cells transiently expressing the YFP protein were visible under a fluorescent microscope, and in this population of fluorescing cells, cell division was very rare. Cells transiently expressing YFP were also apparent in the WUS2 treatment. However, in the WUS2 treatment, cell division was apparent in the cells surrounding the YFP positive cells, resulting in the appearance of a mound of cells with discrete YFP positive cells at the apex. Over the next few weeks these embryo/shoot-like outgrowths continued to elongate with the YFP expressing cells maintaining their position at the apex of projections or a few cells beneath the apex reminiscent of endogenous WUS expression in *Arabidopsis* apical meristems (Mayer et al. (1998) Cell 95:805-815). Embryos and embryogenic calli were transferred every two weeks. After approximately two months only YFP expressing calli were transferred. After 3 months, YFP tissue was transferred to regeneration medium lacking 2, 4-D. No embryogenic YFP positive events were recovered from the control treatment. In contrast, over 24 YFP positive events with embryogenic callus were obtained from the WUS2 treatment. Plants were regenerated from the WUS2 treatment and sent to the greenhouse. This experiment was repeated with similar results, the control transformation frequency was 2%, while the transformation frequency in the In2:WUS2 treatment transformation frequency was 19%.

C. Ectopic Expression of maize WUS2 is sufficient to stimulate organogenesis/embryogenesis in recalicitrant tissues

There exists only a small developmental window in which maize embryos are amenable to tissue culture growth, a prerequisite for transformation. Normally this

occurs between 9-12 days after pollination when the immature embryos are between 1.0-1.5mm in length. Older, larger embryos fail to produce embryogenic callus and thus cannot be transformed. To demonstrate that WUS2 can be used to induce organogenesis/embryogenesis, embryos were isolated 17-18 days after pollination and used for transformation experiments. Using the maize inbreds 581, N46, and P38, immature embryos from greenhouse grown plants were isolated 17-18 days after pollination and cultured on 605J medium. Immature embryos were transformed and visually selected as described in Example 8B. DNA was delivered using co-transformation as described above. As a control, embryos were shot with a 1:1 mixture of plasmid DNA's containing a Ubiquitin driven YFP (Ubi:YFP) and a plasmid containing a Ubiquitin driven uidA gene (Ubi:uidA). In the WUSCHEL treatment the embryos were bombarded with a 1:1 mixture of plasmid DNA's containing the Ubiquitin promoter driving expression of the YFP (Ubi:YFP) and a plasmid containing the WUS2 DNA (SEQ ID NO: 62) driven by the maize Oleosin promoter (Ole:WUS2). Immediately following bombardment embryos were transferred back to low osmoticum 605J medium. Each bombarded plate contained 10 embryos. In the control, each genotype was represented by 2 plates. In the WUS2 treatment, 581 and N46 had 2 plates each while the P38 treatment had only 1 plate (10 embryos). Embryos were observed 5 days after bombardment and hundreds of YFP positive cells were seen in both treatments under the fluorescent microscope. When observed under visible light, embryo-like protuberances were visible in the WUS2 treatment. No embryo-like protuberances were observed in the control treatment. When observed under the fluorescent microscope each of the embryo-like protuberances in the WUS2 treatment was associated with YFP fluorescence demonstrating that WUS2 is sufficient to induce organogenesis from tissues that are normally unresponsive. These embryos are currently being cultured to obtain transformants. As observed in the WUS2 treatment in Example 8B, cell division was

apparent in the cells surrounding the YFP positive cells, resulting in the appearance of a mound of cells with discrete YFP positive cells at the apex (SEE Figure 2).

EXAMPLE 22

Comparison of Wuschel Polypeptide Sequences

The GCG (Accelrys, San Diego, CA) software implementation of the GAP algorithm (Needleman & Wunsch) was used to compare the polypeptides encoded by the full-length genomic or cDNA polynucleotides from maize to known Wuschel polypeptides from the model plant, *Arabidopsis thaliana*. Two *Arabidopsis* polypeptide sequences were used, represented by NCBI GI 4090200 (SEQ ID NO: 25); and NCBI GI 20197404 (which replaced GI 3785979 on 4/18/02). All comparisons were done using default parameters, namely the BLOSUM62 scoring matrix, Gap Creation Penalty = 8, and Gap Extension Penalty =2. All percent sequence identities less than 70% have been rounded up to the nearest whole integer, all others are presented to the nearest tenth of a percent. Table 12 summarizes the results of these comparisons.

TABLE 12: GAP % Sequence Identity to Two *Arabidopsis* Wuschel Polypeptides

SEQ ID NO:	GI 4090200	GI 20197404
27	33%	30%
29	35%	34%
31	41%	33%
33	30%	30%
36	30%	32%
38	32%	33%
40	40%	33%

43	31%	32%
45	33%	34%
47	41%	33%
50	32%	30%
52	38%	35%
54	36%	35%
57	32%	30%
59	38%	35%
61	36%	35%
64	33%	30%
66	39%	35%
68	36%	34%
71	33%	30%
73	39%	35%
75	36%	34%
78	42%	39%
81	29%	35%
88	32%	30%
89	38%	35%
90	36%	35%

The GCG (Accelrys, San Diego, CA) software implementation of the GAP algorithm (Needleman & Wunsch) was further used to compare the polypeptides encoded by the full-length genomic, cDNA, or EST polynucleotides of the present invention. All comparisons were done using default parameters, namely the BLOSUM62 scoring matrix, Gap Creation Penalty = 8, and Gap Extension Penalty =2. All percent sequence identities less than 70% have been rounded up to the

nearest whole integer, all others are presented to the nearest tenth of a percent. Table 13 summarizes the results of these comparisons.

Table 13: GAP % Sequence Identity - Polypeptides

SEQ ID NO:	2	4	6	8	10	12	14	16	18	20	22	24
27	42	34	99.2	99.2	29	53	32	42	36	37	39	34
29	42	34	99.0	99.0	33	53	39	42	32	33	38	35
31	42	33	79.1	79.8	31	53	36	42	34	33	38	35
33	46	28	33	42	32	74.2	93.2	45	37	40	35	34
36	46	36	97.9	97.9	28	52	40	40	36	30	37	33
38	46	36	97.4	97.4	31	52	37	40	33	34	36	34
40	46	34	81.2	80.4	30	52	37	40	35	31	36	35
43	42	34	98.7	98.7	28	53	41	41	36	30	38	34
45	42	34	98.4	98.4	29	53	38	41	33	34	37	35
47	42	32	82.1	81.3	30	53	37	41	35	31	37	35
50	42	34	99.6	99.6	31	53	33	41	36	36	38	34
52	42	35	99.5	99.5	32	53	35	41	33	31	37	35
54	42	31	80.1	80.5	36	53	36	41	35	31	37	35
57	42	34	99.6	99.6	31	53	33	41	36	36	38	34
59	42	35	99.5	99.5	32	53	35	41	33	31	37	35
61	42	31	80.1	80.6	36	53	36	41	35	31	37	35
64	42	34	99.6	99.6	31	53	33	42	36	37	39	34
66	42	35	99.5	99.5	32	53	35	42	33	32	38	35
68	42	31	80.3	80.7	36	53	36	42	34	32	38	35
71	42	34	99.6	99.6	31	53	33	42	36	37	39	34

73	42	35	99.5	99.5	32	53	35	42	33	32	38	35
75	42	31	80.3	80.7	36	53	36	42	34	32	38	35
78	40	26	35	35	32	55	36	43	37	40	41	40
81	41	38	35	35	28	44	38	37	37	34	48	47
88	42	34	99.6	99.6	31	53	33	41	36	36	38	34
89	42	35	99.5	99.5	32	53	35	41	33	31	37	35
90	42	31	80.1	80.5	36	53	36	41	35	31	37	35

The GCG (Accelrys, San Diego, CA) software implementation of the GAP algorithm (Needleman & Wunsch) was further used to compare the full-length genomic, cDNA, or EST polynucleotides of the present invention. All comparisons were done using default parameters, namely Gap Creation Penalty = 50, and Gap Extension Penalty =3. All percent sequence identities less than 70% have been rounded up to the nearest whole integer, all others are presented to the nearest tenth of a percent. Table 14 summarizes the results of these comparisons.

TABLE 14: GAP % Sequence Identity - Polynucleotides

SEQ ID NO:	**1**	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**19**	**21**	**23**
26	42	45	99.3	99.7	44	53	49	50	42	44	44	45
28	41	47	81.8	84.4	44	53	48	50	42	42	37	43
30	38	47	81.8	84.8	45	53	47	50	43	42	36	45
32	47	45	54	53	43	87.4	98.8	47	40	40	43	43
34	41	45	98.9	99.2	42	52	46	48	41	44	38	43
35	41	47	98.9	99.2	44	53	48	49	41	43	44	45
37	46	45	79.7	82.6	48	53	48	49	43	47	37	39

39	39	45	81.5	84.4	44	52	49	49	42	42	38	36
41	43	43	99.2	99.5	42	52	46	47	41	45	38	43
42	43	47	99.2	99.5	43	53	49	48	41	44	44	45
44	46	45	80.3	83.2	47	53	49	48	43	46	37	39
46	39	45	82.1	84.9	44	53	50	48	41	42	38	36
48	39	43	99.6	99.9	43	52	46	47	41	45	39	43
49	39	46	99.6	99.9	45	53	49	48	42	44	43	45
51	47	47	82.5	82.6	44	51	47	48	40	44	39	38
53	41	48	99.5	83.7	45	53	49	48	43	44	40	38
55	39	43	99.6	99.9	43	52	46	47	41	45	39	43
56	39	46	99.6	99.9	45	53	49	48	42	44	43	45
58	47	47	82.5	82.6	44	51	47	48	40	44	39	38
60	41	48	99.5	83.7	45	53	49	48	43	44	40	38
62	39	43	99.6	99.9	43	52	46	49	41	45	40	45
63	39	46	99.6	99.9	42	53	49	50	42	44	39	46
65	47	46	82.7	82.8	43	51	46	50	40	44	40	38
67	41	47	99.5	83.8	44	53	50	50	43	45	40	38
69	39	43	99.6	99.9	41	52	46	49	41	45	38	45
70	39	46	99.6	99.9	45	53	47	50	42	44	44	46
72	47	45	82.7	82.8	43	51	48	50	40	44	38	38
74	41	46	99.5	83.8	45	53	50	50	43	45	36	38
76	47	45	50	49	42	56	45	49	43	44	41	44
77	47	47	55	51	44	53	47	46	41	43	41	41
79	47	40	46	47	39	49	40	49	41	42	45	45
80	47	38	50	51	39	50	40	47	42	43	48	48